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The Inventors of this invention in the sense of being the actual devisers thereof within the meaning of Section 16 of the Patents Act, 1949, are Jorgen Schlichtkrull of 34B, Bellahojvej, Copenhagen, Denmark and Inger Merete Noring of 26 Ornekuldsvej, Charlottenlund, Denmark both subjects of the King of Denmark.

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COMPLETE SPECIFICATION

Improved Process in Crystallization of Insulin

We, Novo Terapeutisk Labortorium A/S, of 115, Fuglebakkevej, Copenhagen, Denmark, a limited liability company, organised under the laws of Denmark, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

Injectable insulin preparations are known the protracted effect of which is exclusively or mainly based on the presence of insulin crystals in aqueous suspension. It is also known that the protracted effect of such aqueous insulin crystal suspensions is to a certain degree dependent upon the size of the suspended insulin cystals.

In the hitherto-known processes of making crystalline insulin nothing has been done to regulate the size of the produced crystals. Besides, it would have had no purpose to undertake such a regulation, as formerly the crystals themselves have not been made constituents of insulin preparations for clinical use.

It is only upon appearance of insulin preparations of practical clinical utility based an aqueous insulin crystal suspensions that the problem arises of how to arrive at crystals of mainly the same size or to obtain the main part by weight of the crystals in sizes within certain determined limits.

The present invention aims at finding a solution of the above mentioned problem. Thus, one of the objects of the invention is to make the insulin crystalline during the crystallization process in the form of crystals of mainly the same size. A further object of the invention is to produce insulin crystals suitable for use as seed material for the production of larger crystals of mainly the same size.

The invention is based on the observation that the presence of freeze-dried amorphous insulin during the crystallization process influences the course of the crystallization as regards the size of the produced crystals and the quantity of crystals of mainly the same size. Here and in the following description the size of the insulin crystals is to be understood as the size in μ of the longest diagonal of the crystal.

The crystallization of insulin is commonly known and very often described in the insulin literature. Though the various crystallization methods may differ somewhat they are, however, based on the same principle, namely, to cause the insulin to crystallize from an aqueous medium by adjusting the p_H-value of the medium to 5 to 7.

Crystallization requires the presence of a crystallization-promoting metal (zinc, cobalt, nickel, cadmium, copper, manganese or iron, among which use is almost made of zinc), and if the insulin itself does not contain such metal in a sufficient amount, the aqueous crystallization medium must be given the necessary content thereof. The necessary amount of crystallization-promoting metal is about 0.4% of the weight of the insulin. In the crystallization in practice usually 2 to 5 times the necessary amount is employed.

In order to fix the p_H value use is generally made of a buffer substance or mixtures of buffer substances. Examples of such buffer substances are acetate, borate, citrate, phosphate, di-ethylbarbiturate and maleate buffers.

It is most common to produce an acid aqueous insulin solution with the necessary metal content, and if desired, buffer substance, and to adjust this solution to

the crystallization *p*H, but it is also possible to precipitate the insulin amorphously in an aqueous medium without the necessary metal content, and then to transform the insulin into crystalline form by adding the necessary amount of metal, for instance in the form of an aqueous solution of a metal salt. Finally, it is also possible to approach the crystallization *p*H from the basic side by using basic insulin solutions.

The present invention relates to a process of the above mentioned kind, according to the above mentioned observation, the characteristic feature of the invention is that the crystallization takes place in the presence of freeze-dried amorphous insulin.

According to one embodiment of the invention the freeze-dried amorphous insulin is added to the insulin-containing crystallization medium after its adjustment to the *p*H-value of the crystallization, but before the formation of the crystals has commenced. According to another embodiment the insulin-containing crystallization medium is produced by mixing an acid aqueous insulin solution and a basic solution containing, if desired, crystallization-promoting metal and buffer substance to obtain the *p*H-value of the crystallization, and adding the freeze-dried amorphous insulin to the basic solution before the mixing process.

Crystallization may take place at *p*H 5 to 7, but it is appropriate to let it take place at *p*H 6.2 to 6.5.

It is preferred to use crystalline insulin or insulin of a similar purity as starting material for the freeze-dried amorphous insulin. The freeze-drying may be carried out in a manner known *per se*. For instance, crystalline insulin dissolved in diluted acid or diluted base may be freeze-dried, the solution having a *p*H-value of for instance 3 or 7.5, respectively, or one may freeze-dried, an insulin solution of a composition corresponding to that of the crystallization medium to which the freeze-dried amorphous insulin is added later-on. It is also possible to freeze-dry a solution of amorphous insulin (free of metals).

Usually clear solutions are freeze-dried, but there is no objection to let a part of the insulin be present in precipitated amorphous form before the freeze-drying.

It has been found that the quantity of freeze-dried amorphous insulin added to the crystallization medium influences the course of the crystallization, it being so that under the same circumstances the crystals will be the smaller the more freeze-dried insulin being added.

Finally, the crystallization medium need not contain insulin beforehand, the desired insulin concentration in the crystallization medium being produced by the addition of the freeze-dried amorphous insulin.

Ordinarily, when using the process according to the invention, insulin crystals are obtained the size of which can be varied from 2 to 7μ dependent upon the amount of freeze-dried amorphous insulin employed, the composition of the freeze-dried amorphous insulin and the crystallization conditions.

Although the crystals or crystal suspensions produced may find therapeutical use they are, however, preferably suitable for use as seed material for the production of larger insulin crystals of uniform size. Their utility for this purpose is not only due to the fact that the crystals possess seed properties, but also that it is possible, when using the process according to the invention, to obtain crystals being completely separated from each other and thus appearing in the form of individual (free) crystal bodies.

Below it will be explained more fully with reference to various examples how the freeze-dried amorphous insulin may be produced and how the crystallization process may be carried out under employment of an addition of the freeze-dried amorphous insulin. It should be noticed that the crystalline insulin used as starting material contains about 0.4% of zinc.

Example 1

500 mgs. of crystalline insulin are dissolved in 50 millilitres of water containing 4 millilitres of 0.1 N hydrochloric acid, and the solution produced is freeze-dried in the usual way under a pressure of about 0.05 mm. of Hg or less.

When adding the said freeze-dried amorphous insulin to an aqueous crystallization medium containing 50 mgs. of citric acid (as sodium citrate per 100 millimetres, 2 mgs. of Zn (as zinc chloride) per 100 millilitres, ½% of insulin, 0.1% of methyl-*p*-oxybenzoate, adjusted to *p*H 6.5, in an amount of 20% of the quantity of insulin in the aqueous crystallization medium, the insulin will crystallize in the form of crystals being uniform in size and form and having a size of about 5μ.

Example 2.

500 mgs. of crystalline insulin are dissolved in 50 millilitres of water containing 4.3 millilitres of 0.1 N hydrochloric acid. This solution is mixed with 50 millilitres of a buffer solution containing 50 mgs. of citric acid, 10 millilitres of 0.1 N sodium hydroxide, 2 mgs. of Zn (as

zinc chloride), 0.16% of methyl-*p*-oxybenzoate, and the *p*H-value of the mixture is adjusted to 6.3, which makes the solution turbid due to precipitation of amorphous insulin. The turbid mixture thus produced is freeze-dried in the usual way under the same pressure as in Example 1.

When adding the said freeze-dried amorphous insulin to the same crystallisation medium as in Example 1 and in the same amount, the insulin will crystallize in the form of crystals of size 5 to 7μ.

EXAMPLE 3

The procedure is the same as in Example 2, with the only modification that the insulin solution which is to be freeze-dried is adjusted to *p*H 6.6 instead of to 6.3, whereby the solution remains clear as no amorphous insulin is precipitated.

The result of the crystallization is insulin crystals of a size of about 2μ.

EXAMPLE 4

The same procedure is followed as in Example 2, with the only modification that the insulin solution which is to be freeze-dried is adjusted to *p*H 7.0. Also in this case the result of the insulin crystallization will be insulin crystals of a size of about 2μ.

EXAMPLE 5

The procedure is as in Example 2, with the only modification that the insulin solution which is to be freeze-dried is adjusted to *p*H 7.5. In this case the result of the crystallization will be crystals of a size of 1 to 1.5μ.

EXAMPLE 6.

500 milligrams of highly purified amorphous insulin with no content of crystallization-promoting metals are dissolved in 50 millilitres of water containing 4.3 millilitres of 0.1 N hydrochloric acid. The solution thus produced is mixed with 50 millilitres of a solution containing 50 mgs. of citric acid, 10 millilitres of 0.1 N sodium hydroxide and 0.16% of methyl-*p*-oxybenzoate whereafter the *p*H-value of the mixture is adjusted to 6.7. The mixture thus produced is freeze-dried in the same manner as in Example 1.

When adding the said freeze-dried amorphous insulin to the same crystallization medium as in Example 1 and in the same amount the insulin will crystallize in the form of crystals of size 5μ.

EXAMPLE 7

500 mgs. of crystalline insulin are dissolved in 50 millilitres of water containing 4.3 millilitres of 0.1 N hydrochloric acid. The solution thus produced is mixed with 50 millilitres of a solution containing 50 mgs. of citric acid, 2 mgs. of zinc (as zinc chloride), 10 millilitres of 0.1 N sodium hydroxide and 0.16% of methyl-*p*-oxybenzoate. The *p*H-value of the mixture is adjusted to about 5.0, whereafter 100 mgs. of insulin freeze-dried as in Example 3 are added and the mixture is agitated. The insulin crystallizes in the form of crystals of size 2.5μ, which are, however, inclined to adhere to each other, which make them less appropriate for use as seed crystals.

EXAMPLE 8

The procedure is as in Example 7, with the only modification that the crystallization takes place at *p*H 5.5 instead of 5.0. The crystals thus produced will have a size of about 2μ and will adhere less to each other than in Example 7.

EXAMPLE 9

The procedure is as in Example 7, with the only modification that the crystallization takes place at *p*H 6.0, by which procedure crystals of size 2μ being completely separated from each other will be obtained.

EXAMPLE 10

The procedure is as in Example 7, except that the crystallization takes place at *p*H 6.3. By this procedure the same result will be obtained as in Example 9.

EXAMPLE 11

The procedure is as in Example 7, except that the crystallization takes place at *p*H 7.0. By this procedure insulin crystals of a size of 1 to 2μ will be obtained, but only a part of the insulin is able to crystallize due to the relatively great solubility of the insulin under the mentioned crystallization conditions.

EXAMPLE 12

500 mgs. of crystalline insulin are dissolved in 50 millilitres of water containing 4.3 millilitres of 0.1 N hydrochloric acid, and the solution thus produced is mixed with 50 millilitres of an aqueous solution containing 178 mgs. of Na_2HPO_4, $2H_2O$, 2 mgs. of zinc (as zinc chloride), 3.8 millilitres of 0.1 N hydrochloric acid and 0.16% of methyl-*p*-oxybenzoate. The *p*H-value of the mixture is adjusted to 6.3, whereafter 100 mgs. of freeze-dried amorphous insulin according to Example 3 are added, and the mixture is agitated until the crystallization is completed. Insulin crystals of a size of about 2μ will be obtained.

EXAMPLE 13

500 mgs. of crystalline insulin are dissolved in 50 millilitres of water contain-

ing 4.3 millilitres of 0.1 N hydrochloric acid, and the solution is mixed with 50 millilitres of an aqueous solution containing 136 mgs. of $CH_3COONa, 3H_2O$, 2 mgs. of zinc (as zinc chloride), 4.5 millilitres of 0.1 N sodium hydroxide, and 0.16% of methyl-*p*-oxybenzoate. The *p*H-value of the mixture is adjusted to about 6.3, whereafter 100 mgs. of freeze-dried amorphous insulin according to Example 3 are added, and the mixture is agitated. The insulin crystals thus produced will have the size of about 2*μ*.

EXAMPLE 14

To 50 millilitres of an aqueous solution containing 50 milligrams of citric acid, 4.5 mgs. of nickel (as nickel chloride), 10 millilitres of 0.1 N sodium hydroxide and 0.16% of methyl-*p*-oxybenzoate, there are added 50 millilitres of an insulin solution having the same composition as that of the insulin solution which according to Example 6 is subjected to freeze-drying, whereafter 100 mgs. of insulin freeze-dried according to Example 3 are added immediately. The *p*H-value of the mixture is adjusted to about 6.2. By the crystallization insulin crystals having a size of about 2*μ* will be obtained.

EXAMPLE 15

To 100 millilitres of an aqueous solution containing 50 mgs. of citric acid, 2 mgs. of zinc (as zinc chloride) and 0.08% of methyl-*p*-oxybenzoate and adjusted to *p*H 6.5 by means of sodium hydroxide, there are added while stirring 600 mgs. of insulin freeze-dried according to Example 3. After stirring for some hours the added insulin has crystallized in the form of crystals having a size of about 2*μ*.

EXAMPLE 16

To 50 millilitres of an aqueous solution containing 50 mgs. of citric acid, 2 mgs. of zinc (as zinc chloride), 10 millilitres of 0.1 N sodium hydroxide and 0.16% of methyl - *p* - oxybenzoate, there are added while stirring 100 mgs. of insulin freeze - dried according to Example 3, and immediately thereafter 500 mgs. of crystalline insulin dissolved in 50 millilitres of water containing 4.3 millilitres of 0.1 N hydrochloric acid, whereafter the *p*H-value of the mixture is adjusted to about 6.0. The crystallization is carried out while stirring and the produced insulin crystals will have a size of about 2*μ*.

EXAMPLE 17

To 50 millilitres of an aqueous solution containing 50 mgs. of citric acid, 2 mgs. of zinc (as zinc chloride) and about 9 millilitres of 0.1 N sodium hydroxide (until *p*H about 11.8) are added 100 mgs. of freeze-dried amorphous insulin produced as described in Example 3.

The freeze-dried amorphous insulin hereby seems to go into solution. Then there are added 50 millilitres of an insulin solution containing 500 mgs. of crystalline insulin, 4.3 millilitres of 0.1 N hydrochloric acid and 0.16% of methyl-*p*-oxybenzoate and *p*H is adjusted to about 6.3.

After the course of some hours crystallization will be completed. The size of crystals is about 2 to 3*μ*.

If the insulin crystals produced according to the above examples are to be used as seed material for industrial production of injectable insulin crystal suspensions containing larger insulin crystals of approximately the same size, it will be appropriate to ensure that no change of the produced seed crystals in the suspension medium will take place during a possible storage. For this purpose such quantity of a crystallization-promoting metal may be added to the suspension medium of the seed crystals that the suspension is stable at *p*H 7, whereafter the mixture is adjusted to this *p*H value. Thus each of the suspensions of insulin crystals produced according to the examples may be diluted in the ratio 1:1 with an aqueous solution containing 50 mgs. of Zn (as zinc chloride) per 100 millilitres and 0.1% of methyl-*p*-oxybenzoate while adding sufficient sodium hydroxide in order to obtain a *p*H value of 7 to 7.5.

In the practical industrial performance of the process the crystallization is usually carried out under sterile conditions so that sterile crystal suspensions are obtained either for direct therapeutical use or for employment in making sterile suspensions of larger insulin crystals for direct therapeutical use.

It is to be understood that the term "freeze-dried amorphous insulin" includes amorphous insulin which is produced by freeze-drying a solution of crystalline insulin.

What we claim is:—

1. A process of crystallizing insulin from an aqueous medium by adjusting the *p*H-value of the medium to 5 to 7, characterised in that crystallization takes place in the presence of freeze-dried amorphous insulin.

2. A process as claimed in Claim 1, characterised in that the freeze-dried amorphous insulin is added to the insulin-containing crystallization medium after its adjustment to the *p*H-value of the crystallization but before formation of the crystals has commenced.

3. A process as claimed in Claim 1 or Claim 2, characterized by producing the insulin - containing crystallization medium by mixing an acid aqueous insulin solution and a basic solution containing, if desired, crystallization-promoting metal and buffer substance, to obtain the crystallization *p*H, and adding the freeze-dried amorphous insulin to the basic solution before the intermixing.

4. A process as claimed in any one of the preceding claims, characterized in that the crystallization takes place at *p*H at 6.2 to 6.5.

5. A process as claimed in any one of the preceding claims, characterized in that the freeze-dried amorphous insulin is produced from crystalline insulin or insulin of a similar purity.

6. A process as claimed in Claim 5, characterized in that the insulin is freeze-dried in a medium of a similar composition to that of the crystallization medium to which the freeze-dried insulin is added.

7. A process as claimed in Claim 1, characterized in providing the insulin content of the crystallization medium by the addition of the freeze-dried amorphous insulin.

8. A process in crystallization of insulin, substantially as hereinbefore described with particular reference to foregoing examples.

9. A suspension of crystalline insulin in the form of single crystals of mainly the same size produced by the process according to any one of the preceding claims.

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